SDF1α-induced chemotaxis of JAK2-V617F-positive cells is dependent on Bruton tyrosine kinase and its downstream targets PI3K/AKT, PLCγ1 and RhoA

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Supplementary information

Cell Culture

Murine 32D and BaF3 cells ectopically expressing Erythropoietin-Receptor (EpoR) and JAK2-WT or JAK2-V617F (32D and BaF3 JAK2-WT/ -V617F), 32D and BaF3 parental cells, and human cell lines (OCI AML3, MOLM 13, HEL and SET2) were cultured in RPMI medium supplemented with 10 % FCS in a humid atmosphere of 5 % CO_2 at 37 °C. 32D JAK2-WT and parental cells (32D and BaF3) additionally received EPO (1 unit (U)/ml of medium) and WEHI-3B conditioned medium respectively.

Antibodies and reagents

The following primary antibodies were used for blotting: P-BTK (Cell Signaling Technology #5082), T-BTK (Santa Cruz #SC-1107), P-p65 (Cell Signaling Technology #3033), T-p65 (Santa Cruz #SC-372), P-AKT (Cell Signaling Technology #9271), T-AKT (Cell Signaling Technology # 9272), P-PLCγ1 (Cell Signaling Technology #2821), T- PLCγ1 (Cell Signaling Technology #2822), Vinculin (Cell Signaling Technology #13901) and GAPDH (Meridian Life Science #H86504M).

Various inhibitors used in the investigation were obtained from Selleck chemicals (ruxolitinib #S1378, ibrutinib #S2680, LY294002 #S1105, U73122 #S8011), and Cytoskeleton (Rho Inhibitor I #CT04-A).

Migration assay

32D JAK2-WT /-V617F or BaF3 JAK2-V617F cells, 32D or BaF3 parental cells, primary T-lymphocytes and granulocytes from healthy donors (HD), JAK2-V617F positive (V617F) and CALR mutated (CALR) MPN patients were serum starved and treated with DMSO or various inhibitors alone or in combination as indicated in the figure legends. 100,000 cells were seeded in the upper compartment and induced to migrate through the transwell inserts (pore size: 3 µm or 5 µm) for 3 hrs. Starvation medium (0.5% FBS) containing EPO (2.5 U/ml) or SDF1α (100 ng/ml) served as chemoattractant's in the lower compartment. Migrated cells were fluorescently labeled with Calcein-AM for 30 mins and quantified using fluorescence microplate reader.

CXCR4 expression

32D JAK2-WT/ -V617F cells were serum starved for 3 hrs and treated with EPO, DMSO and ibrutinib as indicated. Cells were collected, washed and stained with CXCR4 (Biolegend #146505) or IgG (BD #553925) antibodies for 30 mins at room temperature. The samples were washed with PBS before and after staining procedures (3X) and analyzed via flow cytometry.

Actin staining

i. Preparation of slides

32D JAK2-WT/ -V617F cells or primary granulocytes from HD and JAK-V617F MPNs were serum starved for 3 hrs and stimulated with SDF1α (100 ng/ml). Cells were seeded in 4 well chamber slides coated overnight with collagen. Chamber slides were centrifuged at 200 x g for 5 mins and fixed with 4% PFA for 20 mins at room temperature. Cells were permeabilized with 0.1% Triton-X 100, stained with phalloidin-488 (1:1000) for 60 mins at room temperature and mounted with fluoroshield mounting medium with DAPI. The slides were washed with PBS (3X after each step).

ii. Data acquisition and analysis

Images from the chamber slides were acquired with a Zeiss Axiovert 200m using a Plan-Neofluar 40x/0.75. DAPI was excited with 370/40 nm and Phalloidin with 474/28 nm and detected at 425/46 nm and 527/54 nm respectively using Zeiss filter set 62. At least two mosaics of 8 x 10 image set equivalent to 2.5 mm^2 were obtained from every condition. Image analysis was done using CellProfiler (1) to identify the cells and measure the Phalloidin staining intensity. The results for all slides were summarized and visualized using GraphPad.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism Version 5 software (GraphPad Prism, San Diego, CA, USA). Results represent the mean and in some instances median from at least three independent experiments. Statistical significance was analyzed by Student's t-test or Mann-Whitney test as indicated in figure legends. The differences between conditions were considered statistically significant when $p < 0.05$ and denoted by * (*$p<0.05$, ** $p<0.01$,*** $p<0.001$ and **** $p<0.0001$). Data points when not significant, the difference's (Δ) were indicated.

Supplementary figure legends

Supplementary Figure 1: (A) 32D JAK2-WT cells were serum starved for 5 hrs and treated with EPO 2.5 U/ml of starvation medium for various time points as indicated. Whole cell lysates (WCLs) were probed for phosphorylation of PLCγ1 and BTK. Representative immunoblots from independent experiments were shown. **(B)** AML cell lines, OCIAML3, MOLM13, HEL, and SET2 were serum starved for 5 hrs. WCLs were probed for phosphorylation of BTK and p65. OCI AML3 and MOLM13 served as negative and positive controls for BTK expression respectively. Representative immunoblots from independent experiments were shown.

Supplementary Figure 2: (A) 32D JAK2-V617F cells were serum starved for 3 hrs and treated with DMSO or 2 and 5 μM of ibrutinib (IBR 2 and 5 μM) as indicated for an additional 3 hrs. WCLs were probed for phosphorylation of BTK and AKT. Densitometric analysis of P/T-AKT is shown (n=3). **(B)** 32D JAK2-V617F cells growing under steady state condition were treated with DMSO, 0.5 μM of ruxolitinib (RUX 0.5 μM) or 1 μM of ibrutinib (IBR 1 μM) overnight as indicated and WCLs were probed for phosphorylation of PLCγ1 and BTK. Densitometric analysis of P/T-PLCγ1 is shown (n=4). Columns represent mean ± SEM from independent experiments. Statistical significance between different conditions was calculated by Student's t-test; *$p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Supplementary Figure 3: (A and B) 32D JAK2-WT/ -V617F cells were serum starved for 2 hrs and chemotaxis assays were performed using transwells (pore size: 5 μm). Starvation medium (0.5% FBS) containing EPO (2.5 U/ml) or SDF1α (100 ng/ml) either alone (n=4) or in combination (n=5) served as chemoattractant's. **(C)** 32D JAK2-V617F cells were serum starved for 2 hrs and treated with DMSO or 2 μM of ibrutinib (IBR) for an additional 1 hr as indicated and chemotaxis assays were performed using transwells (pore size: 5 μm) (n=4). **(D)** BaF3 JAK2-V617F cells were serum starved for 2 hr and treated with DMSO or 1 μM of ruxolitinib (RUX) or ibrutinib (IBR) for an additional 1 hr as indicated and chemotaxis assays were performed using transwells (pore size: 5 μm) (n=4). Starvation medium (0.5% FBS) containing SDF1α (100 ng/ml) served as chemoattractant. **(E)** 32D JAK2- V617F cells were serum starved for 2 hrs and treated with DMSO or inhibitors targeting BTK (2 μM of ibrutinib: IBR), PI3K (2μM of LY294002) and PLCγ1 inhibitor (0.25 μM of U73122) alone or in combination with IBR as indicated for an additional 2 hrs and chemotaxis assays were performed using transwells (pore size: 5 μm) (n=4). Starvation medium (0.5% FBS) containing SDF1α (100 ng/ml) served as chemoattractant. Columns represent mean ± SEM from independent experiments. Statistical significance between different conditions was calculated by Student's t-test;

*p<0.05, *** p<0.001, and **** p<0.0001. Data points when not significant, the difference's (Δ) were indicated.

Supplementary Figure 4: (A and B) Primary T-Lymphocytes (T-lymphocytes) and 32D parental cells (32D-Par) were serum starved for 2 hrs and pre-treated with DMSO, 1 μM ruxolitinib (RUX) or 2 μM ibrutinib (IBR) and chemotaxis assays were performed using transwells (pore size: 5 μm) (n=5). Starvation medium (0.5% FBS) containing SDF1α (100 ng/ml) served as chemoattractant. **(C and D)** 32D and BaF3 JAK2-V617F cells were serum starved for 2 hrs and treated with DMSO or 1 μM of NFκB inhibitors (BAY 11-7082: BAY and IKK inhibitor VII: IKK) and ruxolitinib (RUX) for an additional 1 hr as indicated and chemotaxis assays were performed using transwells (pore size: 5 μm) (n=3). Starvation medium (0.5% FBS) containing SDF1α (100 ng/ml) served as chemoattractant. **(E)** 32D JAK2-V617F cells were treated with DMSO or different doses of ibrutinib (IBR) or ruxolitinib (RUX) for 48 hrs and assayed for cell viability. Percentage of viable cells (Annexin V and sytox blue negative) were analyzed by flow cytometry (n=4). Columns represent mean ± SEM from independent experiments. Statistical significance between different conditions was calculated by Student's t-test: *p<0.05, ** p<0.01 and *** p<0.001. Data points when not significant, the difference's (Δ) were indicated.

Supplementary Figure 5: (A) 32D JAK2-V617F were transduced with scrambled (Scr) or shRNAs targeting PLCγ1 (#14 and #133) as indicated. After 72 hrs, WCLs were probed for the expression of T-PLCγ1 (left panel). GAPDH served as loading control. Simultaneously, chemotaxis migration assays were performed using transwells (pore size: 5 μm) (n=5). Starvation medium (0.5% FBS) containing SDF1α (100 ng/ml) served as chemoattractant. **(B)** 32D JAK2-WT cells were serum starved for 5 hrs and treated with SDF1α (100 ng/ml of starvation medium) for various time points as indicated. WCLs were probed for phosphorylation of PLCγ1. Vinculin served as loading control. Densitometric analysis of P/T-PLCγ1 is shown (n=3). Representative immunoblots were shown. **(C and D)** 32D parental (32D-Par) and BaF3 parental (BaF3-Par) cells were transduced with scrambled (Scr) or shRNA targeting PLCγ1 (#14) as indicated. After 72 hrs, WCLs were probed for the expression of T-PLCγ1 (left panel). Vinculin served as loading control. Simultaneously, chemotaxis migration assays were performed using transwells (pore size: 5 μm) (n=4). Starvation medium (0.5% FBS) containing SDF1α (100 ng/ml) served as chemoattractant. Columns represent mean ± SEM from independent experiments. Statistical significance between different conditions was calculated by Student's t-test: * p<0.05 and ** p<0.01. Data points when not significant, the difference's (Δ) were indicated.

Supplementary Figure 6: (A) 32D JAK2-WT /-V617F cells were serum starved for 5 hrs and surface CXCR4 expression was analyzed by flow cytometry. 32D JAK2-WT cells (column 2 and 3) were starved in the presence of EPO (1.5 U and 4.5 U/ml). Corrected mean fluorescent intensity (MFI) values for surface CXCR4 were indicated (n=3). **(B)** 32D JAK2-V617F cells were serum starved for 2 hrs and treated with DMSO or 2 μM ibrutinib (IBR) for another 5 hrs. Surface CXCR4 expression was analyzed by flow cytometry and corrected MFI values for surface CXCR4 were indicated (n=4). Columns represent mean ± SEM from independent experiments. Statistical significance between different conditions was calculated by Student's t-test: ** $p<0.01$ and *** $p<0.001$.

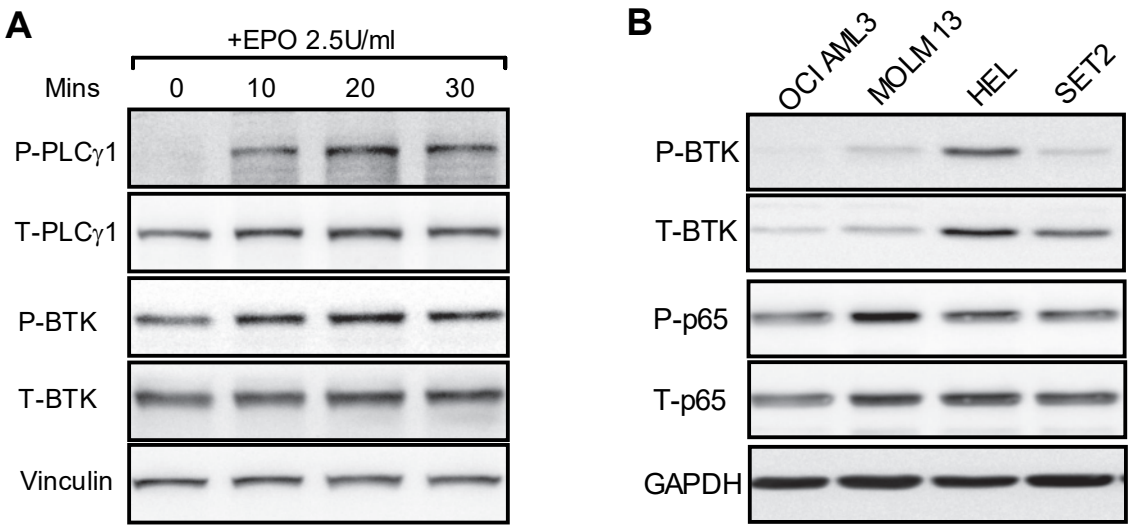
Supplementary Figure 7: RhoA activation assays were performed following the manufacturer's instructions (Cytoskeleton #BK124). **(A)** 32D JAK2-WT cells were serum starved for 4 hrs and treated with EPO (2.5U or 5U) for 30 or 60 mins as indicated and WCLs were analyzed for RhoA activation (n=3). **(B)** Primary granulocytes from five healthy donors (HD) and six JAK2-V617F positive myeloproliferative neoplasms (JAK2-V617F+MPN) patients (MPN) were serum starved for 3 hrs and WCLs were analyzed for RhoA activation. WCLs were additionally probed for total Rho A and Vinculin. Representative blots from 1 HD and 2 MPN (MPN1 and 2) patients were shown. Columns represent mean ± SEM from independent experiments. Statistical significance between different conditions was calculated by Student's t-test; *$p<0.05$. Data points when not significant, the differences (Δ) were indicated.

Supplementary Figure 8: (A) 32D JAK2-WT/ -V617F cells **(B)** primary granulocytes from three healthy donors (HD) and three JAK-V617F MPNs (MPN) were serum starved for 3 hrs and stimulated with SDF1α for another 1 hr as indicated. Collagen coated coverslips with cells were processed as described in the methods section and analyzed for integrated phalloidin intensity relative to control 32D JAK2-WT cells or HD granulocytes. Columns represent mean ± SEM from independent experiments (n=3). Statistical significance between different conditions was calculated by Student's t-test; *$p<0.05$. Data points when not significant, the difference's (Δ) were indicated.

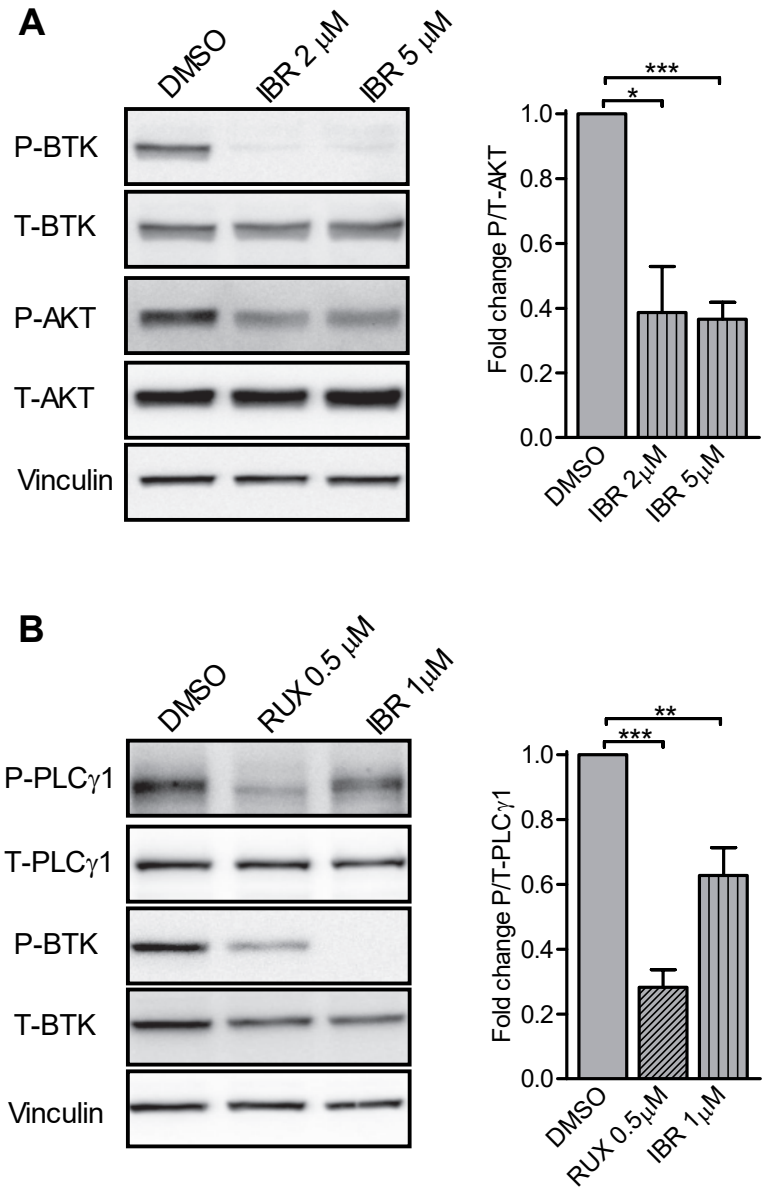
References

1. Carpenter AE, Jones TR, Lamprecht MR, et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome biology. 2006;7(10):R100.

Supplementary Figure 1



Supplementary Figure 2



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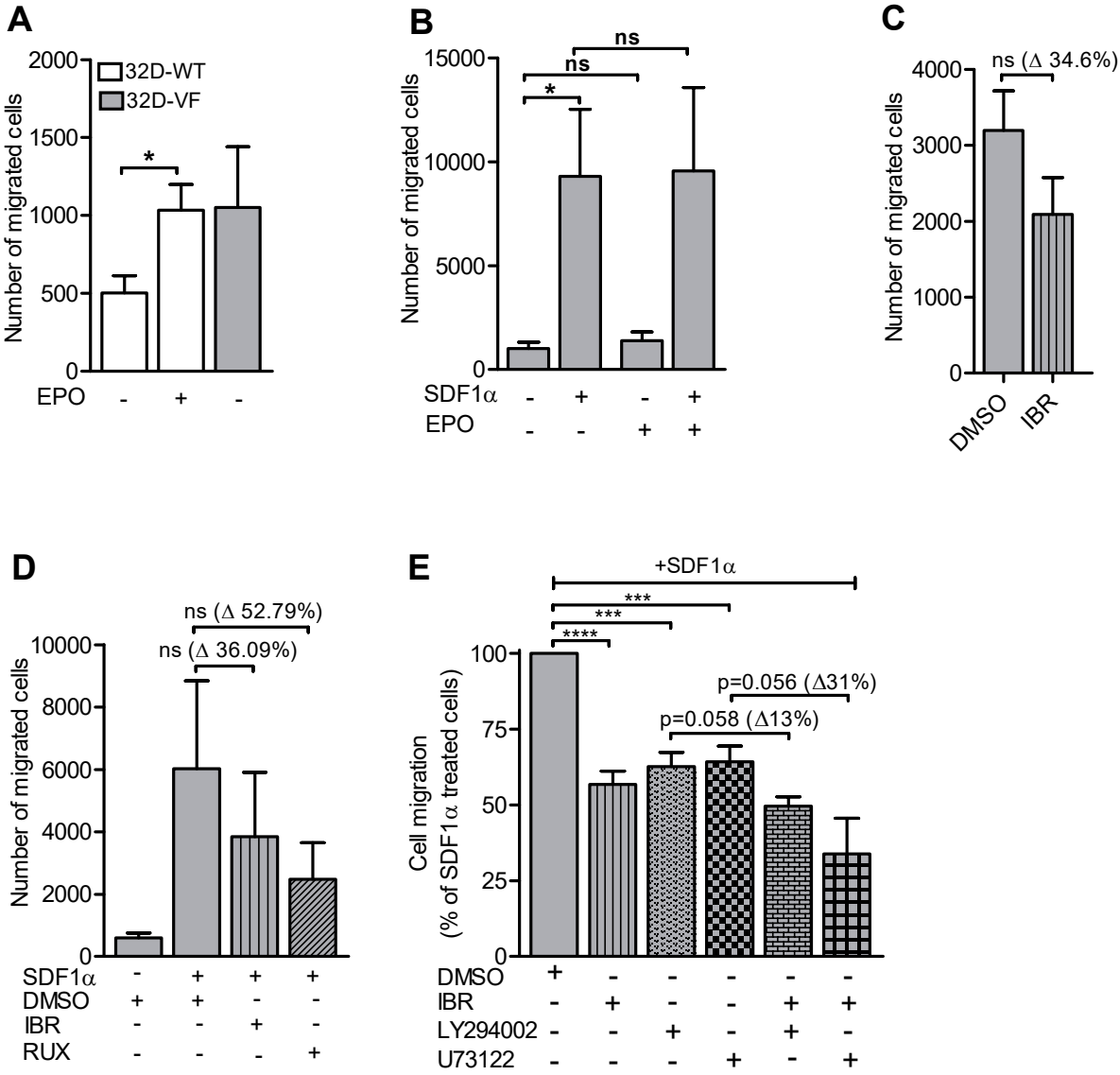

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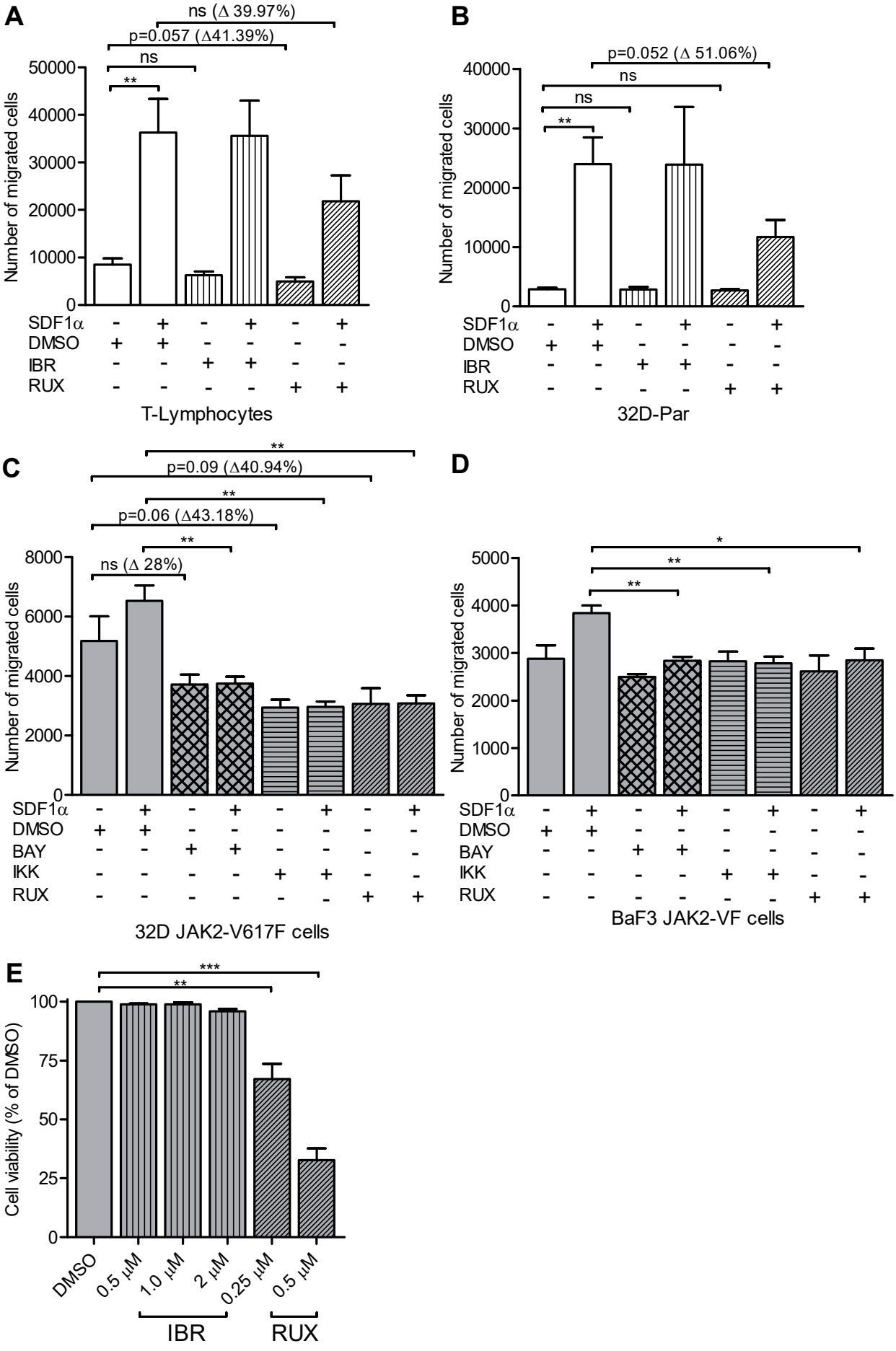

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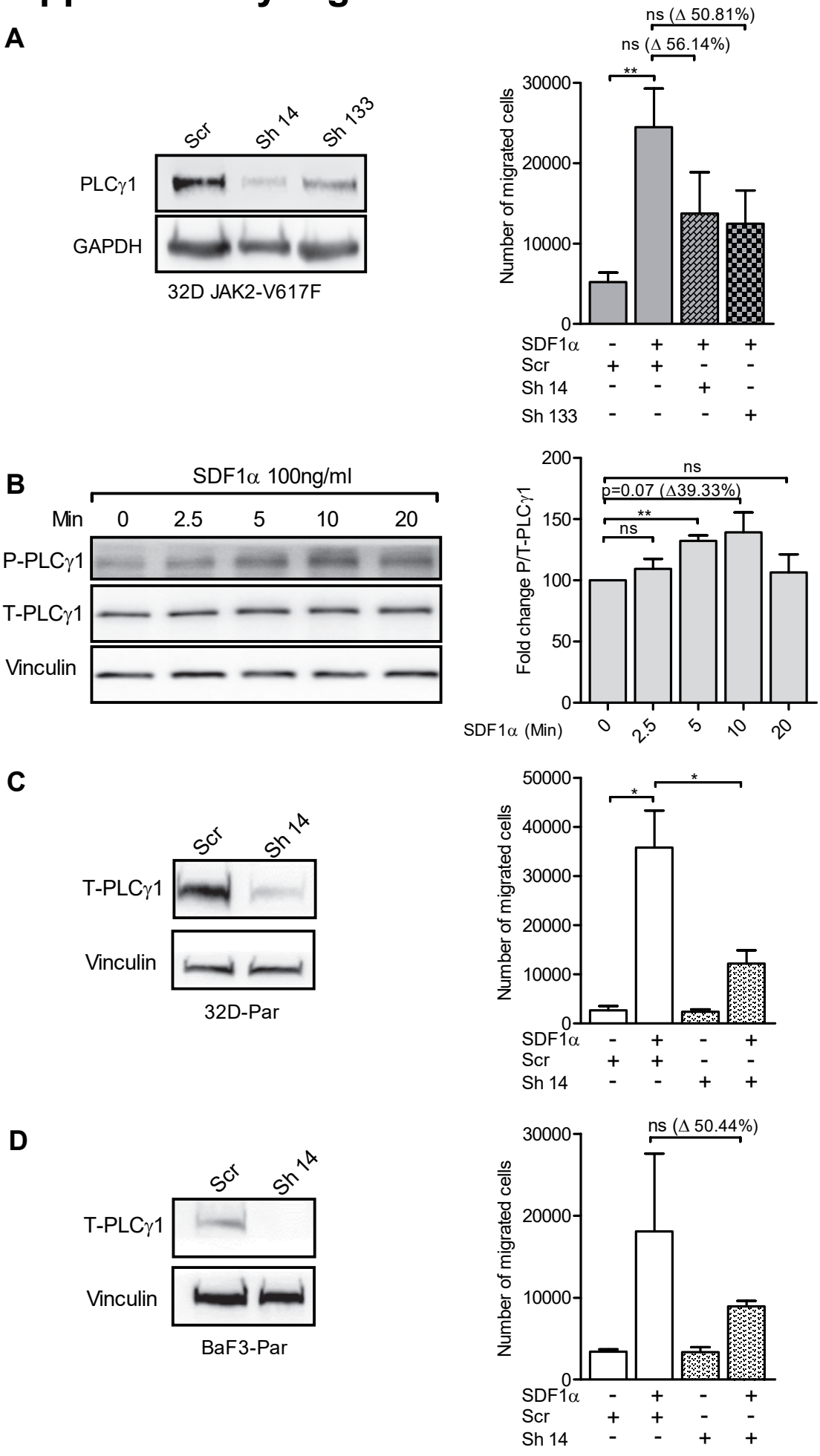

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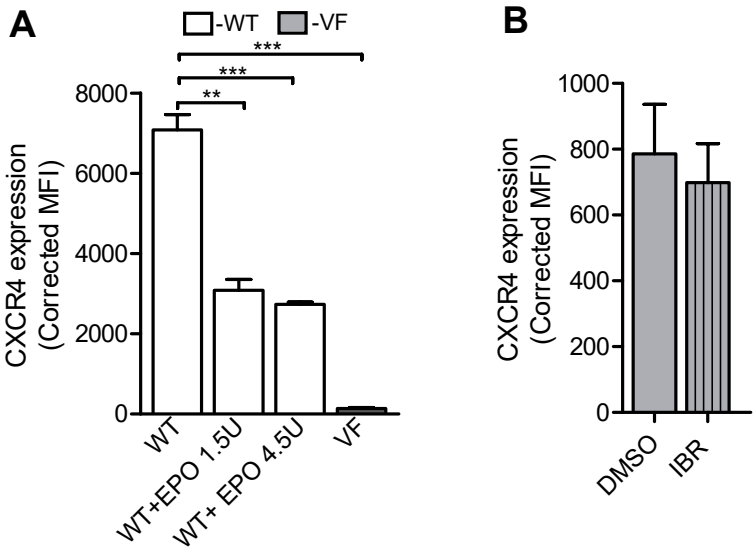

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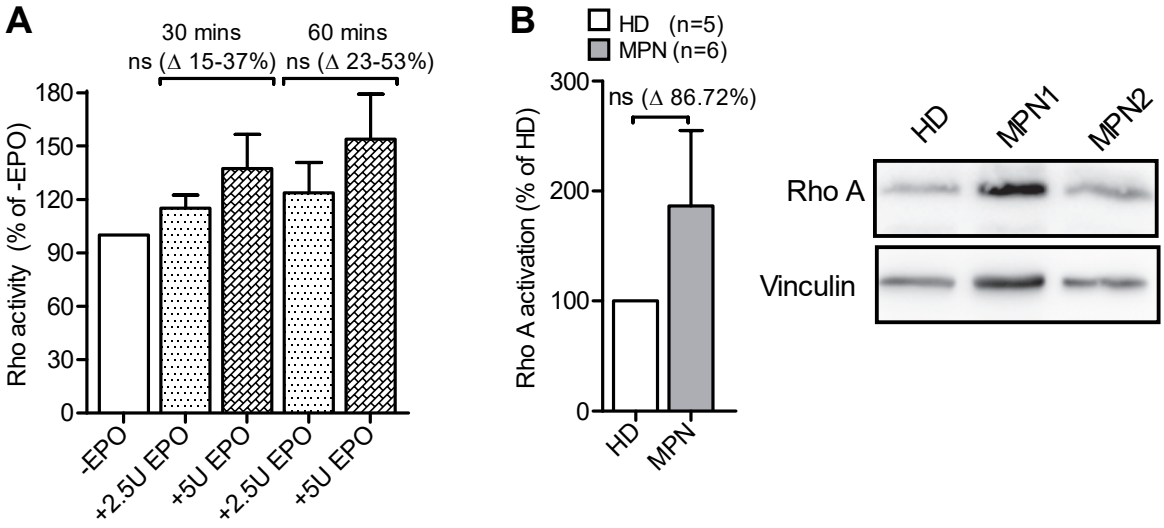

Supplementary Figure 8